The effects of dietary nitrogen on symbiotic nitrogen fixation in the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae)

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ABSTRACT

Wood-feeding termites possess symbiotic bacteria that are capable of fixing atmospheric nitrogen to ammonia. This bacterial process is not only central to termite nutrition, but it also has tremendous ecological importance as fixed nitrogen is often a limiting nutrient in terrestrial ecosystems. However, nitrogen fixation is energetically costly, and we therefore hypothesized that a high nitrogen diet would reduce termite dependence on symbiotic nitrogen fixation. This was tested by rearing workers of the eastern subterranean termite (*Reticulitermes flavipes*) on three food substrates—pine wood, pine needles, and aspen leaves—that differed significantly in percent nitrogen (0.07, 0.36, and 0.93% nitrogen, respectively). Termite and representative food samples were analyzed using an isotope ratio mass spectrometer. A mixed model equation was applied to determine the percent nitrogen derived from the atmosphere within each termite sample. Initial results indicated that nitrogen fixation rates are plastic within *R. flavipes* and they are negatively correlated with dietary nitrogen, as hypothesized.

A follow-up study was conducted to investigate three assumptions associated with the initial experiment. The first assumption is that only nitrogen concentration, and not the nitrogen source (i.e. amino acids, urea, etc.), affects nitrogen fixation rates. This assumption is problematic as previous studies suggest that nitrogen fixation in *R. flavipes* is differentially affected by ammonium nitrate and urea. This is consistent with the observation that ammonium inhibits transcription of the nitrogenase enzyme that is responsible for nitrogen fixation. Second, because the isotope discrimination factor occurring as a result of digestion, Δ_{dig}, is not known for *R. flavipes*, it was estimated by assuming that termites reared on the aspen leaf diet (highest percent nitrogen) did not fix any atmospheric nitrogen. Our estimates of Δ_{dig} ranged from 4.1 to 6.9, which are slightly higher than those reported in the literature for other termite species (~3.4

to 5.8). Third, as the isotope discrimination factor occurring as the result of nitrogen fixation, Δ_{fix}, was never calculated for any termite species, Δ_{fix} was assumed to be within the range of nodulating plants (-2 to 0). The second set of experiments addressed these issues by inhibiting nitrogen fixation in groups *R. flavipes* workers via force feeding the antibiotic kanamicin. Both antibiotic-treated and control (nitrogen-fixing) termites were then exposed to different concentrations of either urea or ammonium nitrate. By comparing the difference between the $\delta^{15}N$ signatures of antibiotic-treated termites and their food sources, we calculated that Δ_{dig} = 2.264. Similarly, by comparing the $\delta^{15}N$ signatures of antibiotic-treated termites with control termites, we determined that Δ_{fix} was equal to -1.125. Using these calculated values for Δ_{dig} and Δ_{fix}, a strong negative correlation between dietary nitrogen and symbiotic nitrogen fixation was evident for termites reared on urea and ammonium nitrate. These studies represent the first attempt to employ stable isotopes to study nitrogen fixation in *R. flavipes* and therefore significantly extend what is known about symbiotic nitrogen fixation and the role that this termite plays in nutrient cycling.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Nitrogen Fixation

Nitrogen is one of the four essential atoms necessary for biological processes, as it is required for protein and nucleic acid synthesis, and it also is a major constituent of several other organic compounds. Yet nitrogen is often limiting in terrestrial ecosystems, which is surprising considering that the Earth's atmosphere is composed of 78% N. Nitrogen limitations are likely the result of several factors, including the nature of the carbon-nitrogen bond, which makes nitrogen difficult to extract from organic compounds, as well as the relative ease with which nitrogen is lost from ecosystems to stream water, and the atmosphere (Vitousek et al., 2002). However, perhaps the most crucial factor in nitrogen limitation is the difficulty associated with transforming atmospheric nitrogen, N_2, to biologically useable forms. N_2 is an incredibly stable molecule, with the two nitrogen atoms held together by a triple bond. Therefore, fixing or converting N_2 to a biologically useful form, such as ammonia, NH_3, is energetically costly.

Although nitrogen can be fixed abiotically, through lightning, natural fires and volcanic activity (Vitousek et al., 2002), the vast majority of nitrogen is biologically fixed by prokaryotic bacteria and Archaea (Nardi et al., 2002). These microorganisms that are capable of fixing atmospheric nitrogen are incredibly taxomically diverse, ranging from the bacterial groups that include the Cyanobacteria and *Pseudomonas* species such as ***P. fluoresens***, to methanogenic Archaea. However, all nitrogen-fixing organisms contain the nitrogenase enzyme which is capable of converting N_2 to NH_3.

Whereas some of these nitrogen-fixing microbes exist as free-living organisms, many form obligate or facultative symbioses with algae, higher plants, or animals. The reasons for this

symbiosis are likely twofold. First, biological nitrogen fixation is an incredibly energetically costly process, and second, a microoxic environment is necessary for optimal nitrogen fixation. This process can require as many as 42 molecules of ATP to fix one molecule of N_2 *in situ* (Slaytor, 2000). Such high energy demands can best be met through the complete oxidation of carbohydrates through aerobic respiration. However, the nitrogenase enzyme is inhibited by high concentrations of molecular oxygen (Hoover, 2000). This inhibition of nitrogenase relates to a second benefit associated with this symbiotic relationship: the multicellular host of nitrogen-fixing prokaryotes can provide a microenvironment with low oxygen concentrations (Nardi et al., 2002). Additionally, symbiotic associations with other organisms can provide nitrogen-fixing microbes with the energy they need. Not surprisingly, microbes that engage in these symbioses are more efficient nitrogen fixers than their free-living congers.

It is also important to note that these symbiotic associations are mutualistically beneficial, in that the multicellular host receives biologically available nitrogen in exchange for energy reserves. In plants and animals whose environment or diet is particularly low in nitrogen, symbiotic associations with nitrogen-fixing bacteria allow them to become the dominant organisms in their environments (Nardi et al., 2002).

1.2 Nitrogen fixation in termites

Termites are a diverse group of hemimetabolous insects that are divided into six major families. The most taxonomically diverse family, Termitidae, comprises the higher termites, all of which lack protists; the remaining five "lower" termite families all possess protists (Breznak, 2000). With such taxonomic diversity, it is not surprising that termites feed on a wide variety of cellulosic materials, such as wood, leaf litter, and soil. While these substrates are diverse in nature, they are generally high in cellulose and lignin, but poor in nitrogen. In fact, the decay-

free wood upon which several species survive contains as little as 0.03-0.7% nitrogen (Tayasu et al., 1994). As the termites themselves are approximately 10% nitrogen (dry weight), this discrepancy has led scientists to postulate that termites must have efficient means both to acquire and retain nitrogen (Holt and Lepage, 2000).

Symbiotic nitrogen fixation in termites represents one of the ways in which these insects compensate for the lack of nitrogen in their diet. Indeed, Tayasu et al.'s (1994) study of the xylophagous *Neotermes koshunensis* (Kalotermitidae) determined that between 30 and 60% of the nitrogen within the termite was derived through symbiotic nitrogen fixation. However, nitrogen fixation is not only nutritionally significant in termites, but is environmentally noteworthy as well. The ability of termites to contribute to the fixed nitrogen supply of their environments is one of the many ways in which termites are ecological keystone species.

In addition to nitrogen fixation, several methods of nitrogen acquisition and conservation have been identified in termites, including behavioral mechanisms such as preferential feeding on substrates high in nitrogen (Prestwich et al., 1980) and recycling nitrogen among colony members via anal and oral trophallaxis (Waller, 2000). Additionally, termites engage in metabolic activities such as lysogenic digestion of symbiotic microorganisms, carbon elimination, and uric acid recycling (Tayasu et al., 1994; Breznak, 2000). All of the latter mechanisms, as well as nitrogen fixation, rely to a varying degree on the termites' prokaryotic symbiotic microbiota, i.e., their bacteria.

1.3 Identification of nitrogen-fixing symbionts

Prior to the advent of molecular techniques such as environmental PCR, identification of the prokaryotic symbionts within the termite hindgut was limited to only those organisms that scientists could successfully isolate and culture. As nitrogen-fixing microbes are especially

difficult to culture due to both their high energy requirements and microoxic needs, little was known about the abundance or diversity of nitrogen-fixing symbionts. In spite of these challenges, French et al. (1976) identified *Citrobacter freundii* as a nitrogen-fixing agent in the guts of the Australian termites *Mastotermes darwiniensis* (Mastotermitidae)*, Coptotermes lacteus* (Rhinotermitidae), and *Nasutitermes exitiosus* (Termitidae). Similarly, Potrikus and Breznak (1977) were able isolate, culture and identify *Enterobacter agglomerans* from the gut of the rhinotermitid *Coptotermes formosanus*.

The development of genetic probes that can reliably test for the presence of specific microbial groups has mediated the study of nitrogen-fixing symbionts in termites, and has led to an appreciation for their diversity. The *nifH* sequence codes for the dinitrogenase reductase enzyme and is highly conserved among diverse nitrogen-fixing microorganisms. Therefore by using the *nifH* sequence as a genetic probe, Ohkuma et al. (1996) were able to identify an incredible diversity of nitrogen-fixing symbionts in the hindgut of the lower termite, *Reticulitermes speratus* (Rhinotermididae). These include a firmicute *Clostridium pasteruanum*, the gamma proteobacteria *Desulfovibrio gigas*, as well as sequences from the Archea domain. A similar study by Kudo et al. (1998) confirmed these results. Additionally, the researchers emphasized that while these findings are useful, the presence of *nifH* sequences does not guarantee that nitrogen fixation is being expressed in the organisms as nitrogenase is regulated both at transcriptional and postranscriptional levels. Additionally, it is possible that the hybridization used in both of these studies did not provide an accurate reflection of the distribution of *nifH* genes in the microbial communities.

1.4 Study of nitrogen fixation in termites

Early studies of nitrogen fixation in termites relied on the acetylene reduction assay (AR). Using this technique, scientists were able to demonstrate nitrogen fixation in termites in the early 1970s (Benemann, 1973; Breznak et al., 1973). This assay relies on the similarity between the triple bond between atmospheric N_2 atoms and that between carbon atoms in acetylene, C_2H_2, inferring that organisms that are able to degrade acetylene to ethylene possess the nitrogenase enzyme that degrades N_2 to $2NH_3$ (Benemann, 1973). Since then, AR has demonstrated that nitrogen fixation occurs in all families of termites (Holt and Lepage, 2000).

While AR is able to both demonstrate and quantify nitrogen fixation, there are several shortcomings associated with the assay. These include the rapid suppression of AR rates following termite disturbance, as well as variation in rates depending on oxygen concentrations in the assay vials (Breznak, 2000; Curtis and Waller, 1995, 1996). These factors likely lead to the underestimation of nitrogen fixation.

A more accurate alternative to AR has been developed which utilizes stable isotopes. This technique compares the natural abundance of ^{15}N in termite tissues to the natural abundance or ^{15}N signature of the termite food sources. These ^{15}N signatures are calculated using the equation (Tayasu et al., 1994): $\delta^{15}N = \left[\frac{\left({}^{15}N/_{14}N \right)sample}{\left({}^{15}N/_{14}N \right)s\tan dard} - 1 \right] * 1000$ where the sample is either the food or termite tissue, and the standard is defined as the ^{15}N ratio of the atmosphere (0.366 atom% ^{15}N). Stable isotopes minimize the manipulation of systems being studied; hence they have become increasingly popular in ecological research and have been used as indicators of food preference as well as nitrogen fixation in termites (Tayasu et al., 1997).

To date, isotope work has focused mainly on the Termitidae including litter-feeding and grass-harvesting termites in Asia and Australia (Holt and Lepage, 2000) and fungus-growers in

Africa (Tayasu et al., 2002). Therefore little is known about the stable isotope signatures of the Rhinotermitidae, which are xylophagous subterranean termites.

1.5 Test Organism

Rhinotermitidae is comprised of several ecologically and environmentally important species, including the eastern subterranean termite, *Reticulitermes flavipes*. This widely-distributed species feeds mainly on wood that is in some form of fungal decay (Breznak, 2000), and is a pest of serious economic importance due to the damage it causes to human structures. Furthermore, *R. flavipes* is characterized by diffuse nests that are closely associated with the soil (Suiter et al., 2002). Based on laboratory and field observations, this species is known to feed on a wide variety of materials, including pine wood, hardwood mulch, cardboard and cellulose paper. Therefore, because of its economic and ecological import, as well as its diversity of food substrates, *R. flavipes* was chosen as a suitable test organism for this study.

1.6 Objectives and Hypotheses

The objectives of this study are to determine if and to what extent dietary nitrogen influences symbiotic nitrogen fixation in the eastern subterranean termite, *Reticulitermes flavipes*. Curtis and Waller's (1997) study suggests that there is not a clear relationship between the concentration of nitrogen in the termites' diet and the rates of nitrogen fixation. However, this finding is problematic given that there is a high energy cost associated with nitrogen fixation. Termite-ingested carbohydrates represent the most likely energy source for symbiotic bacteria. Hence, it seems logical that termites should possess a means to successfully regulate this process such that nitrogen fixation should be inhibited when termites receive an adequate amount of nitrogen from their diets. Perhaps Curtis and Waller (1997) did not observe a negative correlation between dietary nitrogen and symbiotic nitrogen fixation because of their

use of the acetylene reduction assay, which underestimates N fixation. I hypothesize that using stable nitrogen isotopes to study symbiotic N fixation in termites will produce results that reflect the high energy costs associated with symbiotic nitrogen fixation. That is, as dietary nitrogen increases, termite dependence on fixed nitrogen will decrease.

CHAPTER 2: VARIATIONS IN SYMBIOTIC NITROGEN FIXATION FOR TERMITES REARED ON NATURAL SUBSTRATES

2.1 Introduction

Termites are known to feed on a wide variety of cellulosic materials, including soil, highly decayed wood, intact wood, leaf litter, and grass. Additionally, there are minor groups of termite species that feed on fungi, algae and/or lichens (Bignell and Eggleton, 2000). Among these substrates, both the structure and the quality of the nitrogen source varies, such that decay-free wood contains very little nitrogen (0.03-0.7% N or 70-500 C/N ratio) (Tayasu et al., 1994), whereas much higher nitrogen concentrations are found in leaf litter (Prestwich et al., 1980) and soils (~4-10% N) (Cook and Dawes-Gromadzki, 2005). Not surprisingly, nitrogen fixation reflects these trophic variations, such that xylophagous termites have higher nitrogen fixation rates relative to detritus feeders (Breznak, 2000). Furthermore, using the acetylene reduction assay (AR) researchers were able to demonstrate nitrogen fixation in all families of termites and all feeding types except soil feeders (Holt and Lepage, 2000). The authors conclude that the inability of soil feeders to fix atmospheric nitrogen is indicative that they receive adequate amounts of nitrogen from their diets, and therefore do not invest energy in the costly process of nitrogen fixation.

2.2 Objectives and Hypothesis

The objectives of this experiment are to determine if, and to what extent, symbiotic nitrogen fixation is responsive to variations in dietary nitrogen among naturally occurring substrates. Based on the literature, I hypothesize that workers of the eastern subterranean termite, *Reticulitermes flavipes*, that are fed on higher concentrations of dietary nitrogen will be

less dependent on symbiotic nitrogen fixation than termites that are fed on high nitrogen substrates.

2.3 Materials and Methods

2.3.1 Preliminary analyses: The first step in conducting this experiment was to identify natural cellulosic substrates that differed significantly in nitrogen content. Analyses of pine wood, pine needles and aspen leaves revealed that both their nitrogen content and $\delta^{15}N$ signatures were significantly different from each other and the termites (Table 1). Hence, these represented suitable food substrates.

2.3.2 Test organisms: Only workers of the eastern subterranean termite, *Reticulitermes flavipes*, were used in this study, as variations have been observed between members of different castes (Tayasu et al., 1994, 2002; Breznak, 2000). All termites originated from one of three inbred colonies that were established and maintained in Dr. Susan Jones' Extension Entomology Lab (The Ohio State University, Columbus OH, USA). The colonies used were designated with specific codes: A7, B1, and E1. All termites were maintained under ambient laboratory conditions (~22ºC ± 2ºC).

2.3.3 Experimental design: In March 2007 samples of pine wood, pine needles and aspen leaves were ground to be of the same relative form and consistency using either a buzz saw (pine wood) or a Wiley Mill (pine needles and pine leaves). All ground samples were autoclaved and oven dried (60ºC for ~48 hr). Then 0.50 g of each substrate was placed into a plastic cylindrical arena (5 cm diameter, 3.5 cm height; Pioneer Plastics, Inc., Dixon, KY). 1.0 mL of diH_2O was added to each substrate before placing 20 termite workers into an arena which was then capped. Five replicates for each food source and each colony were used, yielding a total of 45 experimental units. Each experimental unit was examined every 48 hours, and dead

termites were recorded and then removed so as to limit cannibalism. Additional water was added as needed.

After one week, termites were transferred to new arenas similarly provisioned with food in order to limit fecal contamination of food substrates. The food samples from both weeks were oven dried (60ºC for ~48 hr) and weighed to calculate termite consumption over the two-week feeding period. Percent mortality was calculated by subtracting the number of surviving termites from the 20 initial termites and dividing it by the initial number of termites. The number of termites cannibalized in each experiment was obtained by comparing the discrepancy between the number of termites that were removed from feeding arenas, and the total mortality within each experimental unit.

2.3.4 Stable Isotope Ratio Mass Spectrometer: Following the two-week feeding period, three termite samples from each colony and feeding treatment (n = 27) were prepared for analysis with an isotope ratio mass spectrometer. Each termite sample contained approximately 5 dried termites (~2 mg) that were pulverized using a ball mill and placed into tin capsules. Representative food samples (n = 3 for each substrate) were similarly prepared, such that each food sample was comprised of ~20 mg. Termite and food samples were then analyzed for natural ^{15}N abundances. These analyses were conducted using a SIRA Series II stable isotope ratio mass spectrometer (VG ISOGAS, Middlewich, UK). Following combustion of the samples in an elemental analyzer (Carlo Erba NA 1500, Milan, Italy), the gaseous emissions were passed to the mass spectrometer using helium as the carrier (Thomas et al., 1991). The isotopic composition, $\delta^{15}N$, was expressed as units of abundance of ^{15}N per mil (‰):

$\delta^{15}N = \left[\frac{\left({}^{15}N/{}_{14}N \right)sample}{\left({}^{15}N/{}_{14}N \right)\text{s tan } dard} - 1 \right] * 1000$ where the standard is defined as the ^{15}N ratio of the atmosphere (0.366 atom% ^{15}N).

2.3.5 Data analysis: To assess the nitrogen fixation rates of termites from each colony that were exposed to each food substrate, Tayasu et al.'s (1994) equation was applied:

$$\%N_{dfa} = \frac{(\delta^{15}N_{wood} + \Delta_{dig}) - \delta^{15}N_{termite}}{(\delta^{15}N_{wood} + \Delta_{dig}) - \Delta_{fix}} * 100,$$ where % N_{dfa} represents the percent nitrogen derived from the atmosphere within each termite sample, and is an indirect measure of nitrogen fixation. Additionally, $\delta^{15}N_{termite}$ is the isotope ratio of one termite; $\delta^{15}N_{wood}$ is the isotope signature of the food source, both of which were directly obtained through measurement with the isotope ratio mass spectrometer. The discrimination factor occurring as a result of digestion, Δ_{dig} is equal to $\delta^{15}N_{termite}$ - $\delta^{15}N_{wood}$. It has been established that this positive factor (~3.5‰ on average for most animals) is a result of the enzymatic discrimination against the ^{15}N isotope that occurs as the result of termite digestion. Thus, over time, the termites tend to excrete more ^{14}N relative to ^{15}N causing their overall $\delta^{15}N$ signature to increase. Finally, Δ_{fix} represents the discrimination factor occurring as a result of nitrogen fixation. This factor is known to be the result of the nitrogenase enzyme's discrimination against ^{15}N. This results in nitrogenase incorporating more ^{14}N relative to ^{15}N, causing a decrease in the $\delta^{15}N$ signature of the termite over time.

As neither Δ_{dig} nor Δ_{fix} are known for *R. flavipes*, both values were estimated in this experiment. It was assumed that termites fed on the aspen leaf diet (highest %N) were not fixing any atmospheric nitrogen, and thus Δ_{dig} could be estimated by subtracting the $\delta^{15}N$ signature of the aspen leaf food source from the $\delta^{15}N$ of the termite. Δ_{fix} was assumed to be between –2 and 0, which are standard values reported for nodulating plants (Tayasu et al., 1994).

Significant differences in food consumption, mortality and cannibalism of termites reared on different food sources and originating from different colonies were determined using one-way

ANOVA ($\alpha = 0.05$). Significant differences in $\%N_{dfa}$ were determined using a Student's *t*-test ($\alpha = 0.05$).

2.4 Results

2.4.1 Termite mortality, food consumption and cannibalism: The average percent mortality of the termites on each food source and from each colony is reported in Table 2. There were no significant differences in percent mortality among termites from different colonies reared on the same food substrate ($p = 0.391$, 0.181, and 0.231 for aspen leaves, pine needles, and pine wood, respectively). However, percent mortality was significantly different for termites reared on different food substrates ($p = 0.006$). Average percent mortality was highest for termites reared on aspen leaves (29.33% ± 5.30%), while termites that were fed pine wood had the lowest percent mortality (12.00% ± 2.48 %).

Termites on average consumed more of the aspen leaf substrate relative to pine needles and pine wood (Table 3), but such differences were not significant ($p = 0.092$). Additionally, differences in consumption among termite colonies were not significant for termites that fed on aspen leaves ($p = 0.546$), although termites from colony D1 consumed significantly more than termites from colonies A7 and E1 on both pine needles ($p = 0.036$) and pine wood ($p < 0.001$).

The average number of termites cannibalized on each substrate and from each colony is reported in Table 4. No significant differences in cannibalism were evident among termites fed on the different food substrates ($p = 0.472$), and there also were no significant differences for the different termite colonies exposed to pine needles ($p = 0.229$), or pine wood ($p = 0.192$). However, termites from colony D1 that were exposed to aspen leaves showed significantly higher levels of cannibalism ($p = 0.030$) than termites from colonies A7 and E1.

2.4.2 $\delta^{15}N$ signatures of termites and food sources: The differences between the $\delta^{15}N$ signature of the termites from each colony and their food source are shown in Figure 1. Termites from colony E1 had significantly lower $\delta^{15}N$ signatures than termites from colonies A7 and D1 ($p < 0.001$). Additionally, the difference between the $\delta^{15}N$ signature of the termite and the $\delta^{15}N$ signature of the food source tends to decrease as the nitrogen content of the food decreases.

2.4.3 $\%N_{dfa}$ in termites vs. dietary nitrogen: To compare the effects of dietary nitrogen on symbiotic nitrogen fixation, the percent N derived from the atmosphere within each termite sample, $\%N_{dfa}$, is plotted against the nitrogen content of the termites' food source (Figure 2). In doing so, the $\%N_{dfa}$ values for termites exposed to pine wood (0.07% N) are aligned at the left of the graph. The $\%N_{dfa}$ for termites exposed to pine needles (0.36% N) are represented at the middle of the x-axis. Termites exposed to aspen leaves (0.93% N) were assumed to not fix any atmospheric nitrogen ($\%N_{dfa} = 0$).

Termites from colony E1 fixed significantly more nitrogen ($p = 0.040$) than termites from colonies A7 and D1 (Figure 2). Contrary to the hypothesis, termites from colony E1 also showed a slight increase ($p = 0.64$) in nitrogen fixation as dietary nitrogen increased from pine wood to aspen leaves. As expected, termites from colonies A7 and D1 demonstrated a decrease in nitrogen fixation as dietary nitrogen increased. This negative correlation was nonsignificant for termites in colony D1 ($p = 0.59$), but it was significant for termites in colony A7 ($p = 0.005$).

2.5 Discussion

The observation that mortality was significantly higher among termites exposed to aspen leaves, the highest nitrogen substrate, was unanticipated. Indeed, throughout the course of the experiment, the aspen leaf treatment was more prone to fungal infestations, which could have contributed to the higher observed mortality. This trend also could have been the result of the

termites' microbiota being ill-adapted to digest aspen leaves, as *R. flavipes* does not naturally feed on leaf litter. Indeed, termite workers exposed to pine wood, the food on which they were initially reared, suffered the lowest mortality, which is consistent with this explanation.

However, it is interesting to note that overall food consumption was not significantly different for *R. flavipes* workers maintained on aspen leaves, pine needles, and pine wood. This is somewhat surprising because if *R. flavipes* were truly ill-equipped to feed upon aspen leaves, significantly lower levels of consumption would be expected on these substrates. Future studies should address this by first trying to limit fungal growth, and also by using other indicators of termite health besides mortality. For example, the change in average termite weight from the beginning of the experiment to the end could be a useful measure of food preference and termite vitality.

Additionally, future studies should also attempt to limit cannibalism of dead or injured nestmates, perhaps with 24 hr rather than 48 hr observations to remove dead or dying termites. Although cannibalism was not significantly different among termites exposed to the different food sources, any cannibalism is problematic. This is because termites represent a very rich nitrogen source which was unaccounted for in the initial calculations. Furthermore, anal and oral trophallaxis among nestmates also contributes an additional source of nitrogen to the termites' diet. Perhaps the best way to eliminate these factors in future studies is to maintain termites in separately. However, as termites are eusocial insects that rely on nestmates, it is likely that termites maintained individually will not fare well.

In spite of these difficulties, this study demonstrated that nitrogen fixation rates are plastic within *R. flavipes* and that nitrogen fixation is responsive to changes in dietary nitrogen. As this is the first time nitrogen fixation in *R. flavipes* has been studied using stable isotopes, this

study significantly extends what is known about nitrogen fixation plasticity in this species. Contrary to the results reported by Curtis and Waller (1997), this experiment demonstrates that nitrogen fixation rates are responsive to nitrogen variations in natural substrates. However, the results of this experiment did not conclusively demonstrate the expected negative correlation between dietary nitrogen and symbiotic nitrogen fixation; only *R. flavipes* workers from colony A7 showed a significant decrease in symbiotic nitrogen fixation as dietary nitrogen increased, while termites from colonies D1 and E1 showed no significant change in nitrogen fixation as dietary nitrogen increased.

The variation in nitrogen fixation rates observed among colonies A7, D1, and E1 in this experiment is not surprising as termites from different colonies are known to possess different bacterial assemblages. Indeed, a study conducted by Matsuura (2001) suggests that these differences in termite microbiota mediate nestmate recognition in the lower termite, *Reticulitermes speratus* (Rhinotermitidae). Additionally, the lower $\delta^{15}N$ signature of termites from colony E1 could possibly be the result of higher concentrations of uric acid within these individuals. It was observed that termites from this colony were chalky white in appearance, which is indicative that they had higher concentrations of uric acid. As a study by Tayasu et al. (2002) suggests, increased concentrations of uric acid are negatively correlated with $\delta^{15}N$ signatures. Therefore the higher rates of nitrogen fixation that were calculated for termites from this colony could be an artifact of the depleted $\delta^{15}N$ of these termites as a result of their higher uric acid content. However this reasoning cannot explain why symbiotic nitrogen fixation did not decrease in colony E1 termites as the nitrogen content of their diet increased.

The subsequent experiment (Chapter 3) seeks to remedy some of the potential sources of experimental error associated with this study. In doing so, the negative relationship between

dietary nitrogen and symbiotic nitrogen fixation in termites should become more apparent. Additionally, an accurate understanding of how symbiotic nitrogen fixation is influenced by changes in dietary nitrogen in *R. flavipes* will not only be beneficial for future studies of symbiotic nitrogen fixation in all termites, but also will be useful to additional studies concerning the ecology of *R. flavipes* such as food preferences, and estimations of the amount of fixed nitrogen that this termite species contributes to its environment.

Table 1. Nitrogen concentration (%N) and ^{15}N signatures ($\delta^{15}N$) of selected food substrates and termite workers pooled from three *R. flavipes* laboratory colonies.

Sample ID	%N	$\delta^{15}N$
Aspen leaves	0.93	-5.1
Pine needles	0.36	-0.10
Pine wood	0.07	0.10
Termites	9.99	0.93

Table 2. Percent mortality (X ± S.E.) of *R. flavipes* workers from three lab colonies (A7, D1, and E1) exposed to aspen leaves, pine needles and pine wood as food substrates

Colony	***Food Substrate***		
	Aspen Leaves	**Pine Needles**	**Pine Wood**
A7 (n = 5)	24% ± 4.30	21% ± 6.20	10% ± 2.24
D1 (n = 5)	40% ± 12.25	21% ± 1.87	18% ± 6.25
E1 (n = 5)	24% ± 9.14	11% ± 2.92	8% ± 2.55
Overall average (n = 15)	29.33% ± 5.30	17.67% ± 2.53	12% ± 2.48

Table 3: Consumption (g) of aspen leaves, pine needles, and pine wood (X ± S.E.) during a 2-wk period by *R. flavipes* workers from three experimental colonies

Colony	***Food Substrate***		
	Aspen Leaves	**Pine Needles**	**Pine Wood**
A7 (n = 5)	0.041 ± 0.008	0.023 ± 0.002	0.014 ± 0.001
D1 (n = 5)	0.035 ± 0.003	0.040 ± 0.006	0.040 ± 0.004
E1 (n = 5)	0.032 ± 0.004	0.028 ± 0.004	0.022 ± 0.002
Overall Average (n =15)	0.036 ±.003	0.032 ± 0.011	0.024 ± 0.005

Table 4: Number of *R. flavipes* workers (X ± S.E.) per colony that were cannibalized during the 2-wk exposure period

Colony	***Food Substrate***		
	Aspen Leaves	**Pine Needles**	**Pine Wood**
A7 (n = 5)	0.8 ± 0.37	1 ± 0.32	1.6 ± 0.51
D1 (n = 5)	2.6 ± 0.81	2 ± 0.63	3 ± 1.14
E1 (n = 5)	0.4 ± 0.24	1 ± 0.32	1 ± 0.32
Overall Average (n = 15)	1.27 ± 0.38	1.33 ± 0.27	1.87 ± 0.46

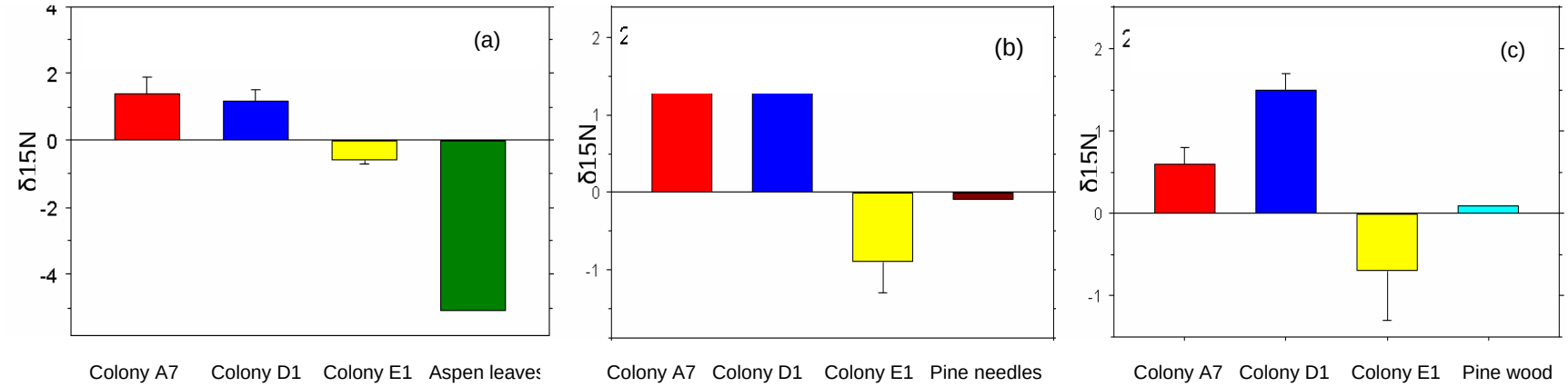

Figure 1: The average δ15N values of termites from colonies A7, D1, and E1 on their respective food sources: (a) aspen leaves, (b) pine needles, and (c) pine wood. Vertical bars indicate one standard error. The graphs are arranged such that the % N of the food source decreases from left to right.

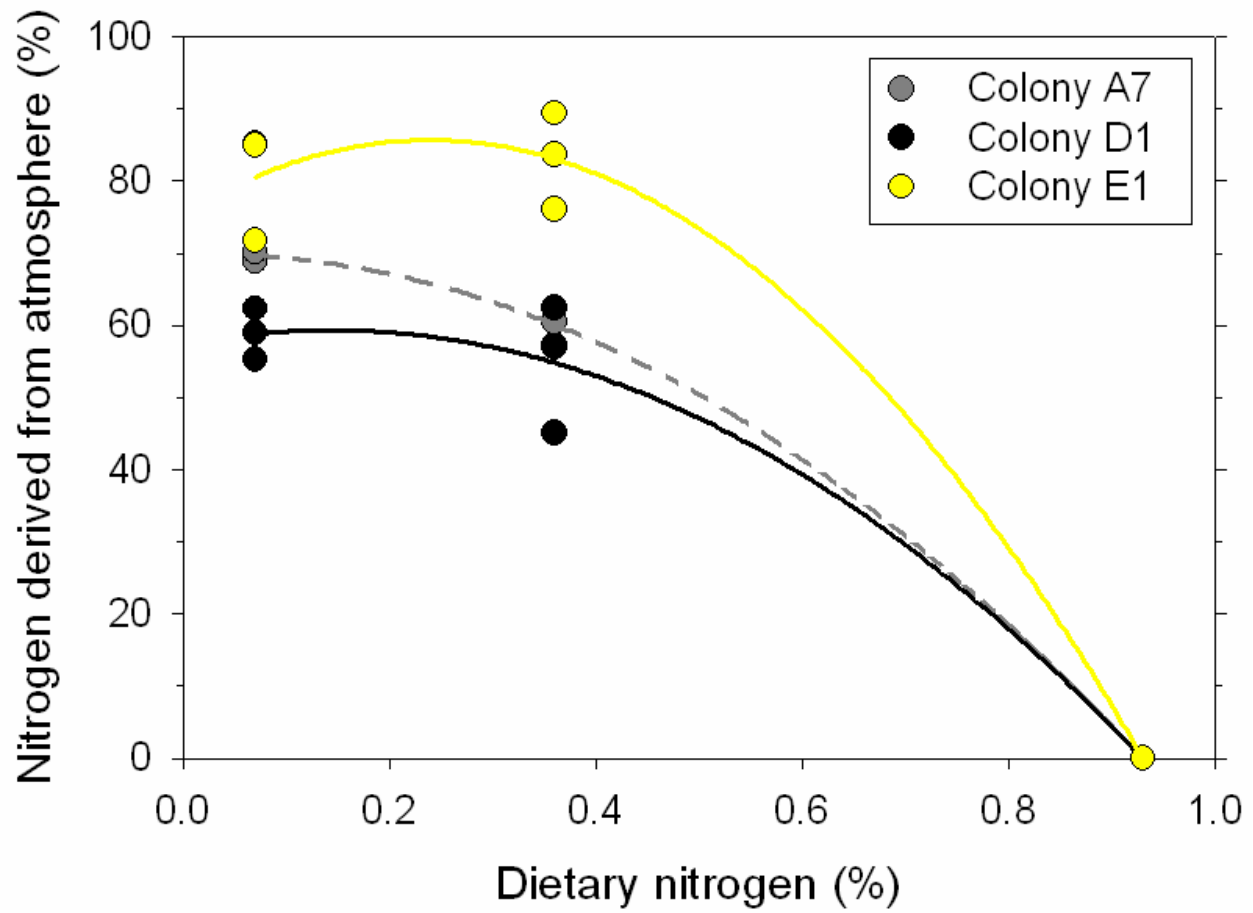

Figure 2: Percent nitrogen derived from the atmosphere for termites (%N_{dfa}) vs. percent nitrogen of food source. Data points to the left of the graph where % dietary N = 0.07 correspond to termites reared on pine wood, and data points that align with % dietary N = 0.36 correspond to termites that were fed pine needles. All fixation values for termites fed on aspen leaves assumed to be 0.

CHAPTER 3: VARIATIONS IN SYMBIOTIC NITROGEN FIXATION IN TERMITES REARED ON CONTROLLED NITROGEN SOURCES

3.1 Introduction

While the initial study (Chapter 2) suggested that nitrogen fixation rates in *Reticulitermes flavipes* are plastic and responsive to changes in dietary nitrogen among naturally occurring substrates, the results were not as compelling as expected. These somewhat inconclusive results are likely associated with three fundamental assumptions made in the initial experiment (See Chapter 2).

The first assumption that needs to be addressed is that only the percent nitrogen, and not the source of the dietary nitrogen, affects symbiotic nitrogen fixation. This is problematic as a study conducted by Curtis and Waller (1997) demonstrated that nitrogen fixation was successfully inhibited in *Reticulitermes* spp. when worker termites were fed filter paper treated with ammonium nitrate or mixtures of the amino acids, proline, tryptophan and leucine. However, no change in nitrogen fixation was observed when *Reticulitermes* workers were fed filter paper treated with ammonium phosphate, urea, or a mixture of the amino acids, histidine, serine, and aspartic acid. While the authors were unable to explain why certain amino acids inhibited N fixation and others did not, they suggested that amino acids may be sources of NH_3. As it is known that high concentrations of NH_3 and the ammonium ion, NH_4^+, inhibit transcription of the nitrogenase enzyme (Hoover, 2000), this can explain why both NH_4^+ and certain amino acids more successfully inhibit nitrogen fixation than other nitrogen sources. These studies therefore suggest that the source of nitrogenous compounds can impact nitrogen fixation rates in termites. As aspen leaves, pine needles and pine wood likely contained variable

amounts of different nitrogenous substrates, these differences could have contributed to the inconclusive results observed in the initial experiment reported in Chapter 2.

A second aspect of the initial study that is problematic is the assumption that termites fed the aspen leaf diet did not fix any atmospheric nitrogen. Although making this assumption was necessary to estimate the value of Δ_{dig}, doing so produced estimates of Δ_{dig} (4.1-6.7) that were slightly higher than those reported for other termite species (~3.4-5.8) (Cook and Dawes-Gromadzki, 2005). As Δ_{dig} greatly impacts the calculation of the percent nitrogen derived from the atmosphere for termites exposed to a particular treatment, future studies should aim to inhibit symbiotic nitrogen fixation in *R. flavipes*. This could be mediated either through treating termites with antibiotics to eliminate their nitrogen-fixing symbionts, or possibly by increasing the oxygen content of the termites' environment to inhibit nitrogenase activity (Hoover, 2000).

Additionally, the assumption that Δ_{fix} for termites is within the same range as that found in nodulating plants is potentially problematic. Indeed, both the composition of nitrogen-fixing microbes as well as their arrangement in the termite gut are very different from legume symbionts and their arrangement in the root nodule (Kudo et al., 1998). Because Δ_{fix} also has a great impact on the calculated rates of the $\%N_{dfa}$ within each termite sample, an accurate calculation of this term is necessary.

3.2 Objectives and Hypotheses

The objectives of this study were to address the fundamental assumptions associated with the initial study to determine if and to what extent variations in both nitrogen source and nitrogen content affected symbiotic nitrogen fixation in the eastern subterranean termite, *Reticulitermes flavipes*. To assess these effects, termite workers were reared on different concentrations of either ammonium nitrate or urea. I hypothesized that there would be a negative correlation

between symbiotic nitrogen fixation and dietary nitrogen for termites reared on both ammonium nitrate and urea sources. However, given the role that ammonium plays in inhibiting nitrogen fixation (Hoover, 2000), I anticipated that the correlation would be stronger for *R. flavipes* workers on an ammonium nitrate diet as compared to a urea diet.

Additional objectives of this study were to accurately determine Δ_{dig} and Δ_{fix} for *R. flavipes.* To do so, nitrogen fixation was inhibited in 50% of the *R. flavipes* workers via an antibiotic treatment to eliminate their nitrogen-fixing symbionts. By subtracting the $\delta^{15}N$ value of the food source from that of the $\delta^{15}N$ signature of the non-nitrogen-fixing termites, I hypothesized that an accurate calculation of Δ_{dig} could be obtained. Similarly I anticipated that Δ_{fix} could be calculated by comparing the difference in $\delta^{15}N$ signatures of termites possessing nitrogen-fixing symbionts (controls) versus those lacking the symbionts (treatment). In this case, I expected the $\delta^{15}N$ signatures of the "nitrogen-fixing" (control) termites to be lower than those of "non-fixing" (antibiotic-treated) conspecifics, yielding a negative value for Δ_{fix}.

3.3 Materials and Methods

3.3.1 Preliminary antibiotic treatments: Based on the species of nitrogen-fixing bacteria that have been identified in *Reticulitermes flavipes* and other termite species (Ohkuma et al., 1996; Kudo et al., 1998), the broad-range antibiotic kanamicin was selected to inhibit symbiotic nitrogen fixation in the termites. To determine the appropriate dosage that would eliminate nitrogen-fixing symbionts while limiting termite mortality, three groups of 75 termites were fed on a cellulose pad treated with either 0, 30, 100, 300, 500 or 1,000 ppm kanamicin for two weeks.

Surviving termites were analyzed using the acetylene reduction assay to determine the amount of nitrogen fixation. To do so, groups of approximately 20 termites/antibiotic

concentration replicate were placed in 7 mL glass screw-capped vials with butyl rubber septa. Approximately 1 mL of the headspace was replaced with acetylene gas using a gastight syringe. This resulted in a final atmosphere of approximately 14% acetylene. The samples were incubated at 25ºC-30ºC for ~3 hr, then a 1 mL sample of the headspace in the vials was removed for analysis using a Varian CP-3800 gas chromatograph equipped with an FID and HaysepR packed column (Varian, Walnut Creek, CA) (Langston et al., 2005). This provided a measure of the ethylene concentration within the vials. Ethylene production rates were calculated and converted to nitrogen fixation rates based on N weight and using a constant of acetylene reduction to N fixation (= 3) (Bentley, 1984). The results, which are shown in Figure 3, revealed that nitrogen fixation rates associated with *ad lib* feeding on the antibiotic treatment were highly variable. This high degree of variability likely was the result of termites' differential feeding on the antibiotic-treated cellulose pads.

3.3.2 Force feeding of antibiotic treatments: A follow-up study was conducted wherein termites were force fed antibiotic solution. A 100 nL droplet of either an 800 or 1000 ppm kanamicin solution was placed on the mandibles of each *R. flavipes* worker using a microapplicator. Each solution was marked with Nile Blue dye in order to assess consumption of the antibiotic droplet. Termites were observed under a dissecting microscope to ensure that the drop had been ingested, which was indicated by the presence of very small dot of blue coloration in their foregut. For each concentration, 40 termites were placed in an arena with a moist cellulose pad and held for two weeks. Three replicates were established for each antibiotic treatment. One control group (3 replicates; 40 workers/rep) was similarly handled and force fed a 100 nL droplet of Nile Blue dye and diH_2O solution.

Nitrogen fixing capability was assessed using the acetylene reduction assay; procedures are outlined in 3.3.1. These results (Figure 4) indicated that manually force-feeding kanamicin to the termites was effective in eliminating symbiotic nitrogen fixation. Therefore this methodology was adopted for subsequent antibiotic treatments.

3.3.3 Experimental design: To assess both the effects of nitrogen source as well as nitrogen concentration on symbiotic nitrogen fixation, five different nitrogen diets with variable amounts of nitrogen were used: 0% N, 0.1% and 1.0% N originating from urea, and 0.1% and 1.0% N originating from ammonium nitrate. To generate these different treatments, 1 mL of prepared solutions of either distilled water, urea, or ammonium nitrate was applied to cellulose pads. All solutions also contained Nile Blue dye as a marker that allowed visual confirmation that termites fed on their respective food sources.

Ten replicates of each diet were prepared, of which 50% received antibiotic-treated termites and the remainder received control termites that retained their nitrogen-fixing symbionts. The antibiotic-treated termites had been force fed a 100 nL droplet of a 1000 ppm kanamicin + Nile Blue dye solution, prior to their placement in a plastic arena (n = 5 replicates; 28 termites/rep). The control termites for each food diet had been force fed a 100 nL droplet containing Nile Blue dye only (n = 5 replicates; 28 termites/rep). All termites originated from the same inbred lab colony, B1. As in the previous experiment (Chapter 2), each replicate was examined approximately every 24 to 48 hours, and dead termites were removed to limit cannibalism. The number of the dead termites was recorded, and water was added to each replicate as necessary.

Approximately two weeks after introduction to the experimental nitrogen source, the surviving termites in each replicate were collected. The ability to fix atmospheric nitrogen was

assessed using AR, using the same procedure previously described in section 3.3.1. Percent mortality was calculated for termites in each replicate as previously described in section 2.3.3. Additionally, consumption was calculated by subtracting the final weight of the cellulose pad from its initial weight.

3.3.4 Isotope ratio mass spectrometer analysis: Immediately following AR analysis, termites were frozen at 0ºC (24 hr) and dried at 60ºC for 48-72 hr. Representative food samples and all termite samples were prepared for analysis with a Thermo Delata Plus isotope ratio mass spectrometer (IRMS) interfaced to a NC2500 elemental analyzer at the Cornell Isotope Laboratory. As in the previous study (Chapter 2), analysis with the IRMS provided output of the $\delta^{15}N$ signatures of termites and their food sources.

3.3.5 Data analysis: To assess the nitrogen fixation rates of termites from each food treatment, Tayasu et al's equation again was applied: $\%N_{dfa} = \frac{(\delta^{15}N_{wood} + \Delta_{dig}) - \delta^{15}N_{termite}}{(\delta^{15}N_{wood} + \Delta_{dig}) - \Delta_{fix}} * 100$ (1994). The values of Δ_{dig} and Δ_{fix} were calculated based on the observed $\delta^{15}N$ signatures of termites treated with antibiotics, as described in section 3.2.

Differences in the percent nitrogen derived from the atmosphere within control termites reared on each nitrogen diet were determined using both a student's *t*-test and one way ANOVA. Similarly, differences in consumption, mortality and cannibalism between antibiotic-treated and control termites reared on the same food substrate were assessed using a student's *t*-test. Overall differences in consumption, mortality and cannibalism for termites on all different food substrates were evaluated using one way ANOVA. In all of these cases, $\alpha = 0.05$.

3.4 Results

3.4.1 Consumption, mortality and cannibalism: Following the two week feeding period, all termites had a blue tint, indicating that they had consumed the cellulose pad

containing the respective nitrogen treatment. Average consumption of the food substrates is shown in Table 5. Consumption was significantly higher among termites that had been treated with antibiotics (non-fixing) compared to the controls for three of the food diets: 0% N ($p = 0.001$), 0.1% N urea ($p = 0.005$), and 0.1% N ammonium nitrate ($p = 0.052$). However, no significant differences in consumption were seen between nitrogen-fixing and non-fixing termites reared on either 1% N urea ($p = 0.068$) or 1% N ammonium nitrate ($p = 0.503$). For termites exposed to both of these 1% N diets, the control termites tended to consume more food relative to antibiotic-treated termites. Consumption rates among all of the N diets, however, were significantly different ($p = 0.007$), such that termites that fed on the lower nitrogen substrates (0% N, 0.1% N urea, and 0.1% N ammonium nitrate) consumed significantly more than termites reared on the higher nitrogen diets (1% N urea and 1% N ammonium nitrate).

Surprisingly, no significant differences in percent mortality were observed, either between nitrogen-fixing (control) and non-fixing (antibiotic-treated) termites reared on the same substrate: 0% N ($p = 0.375$), 0.1% N urea ($p = 0.180$), 0.1% ammonium nitrate ($p = 0.353$), 1% urea ($p = 0.198$), and 1% ammonium nitrate ($p = 0.084$). However, there was a significant difference in percent mortality among termites exposed to different N diets ($p < 0.001$). As in the previous experiment (Chapter 2), mortality was higher among termites reared on the higher nitrogen substrates relative to those that had been reared on the lower nitrogen food sources (Table 6).

No significant differences in cannibalism were observed for termites reared on different nitrogen diets ($p = 0.109$; Table 7). Additionally, there were no significant differences in cannibalism between nitrogen-fixing and non-fixing termites reared on the same nitrogen diet

(0% N, p = 0.749; 0.1% N urea, p = 0.241; 0.1% N ammonium nitrate, p = 0.273; 1% N urea, p = 0.081; and 1% N ammonium nitrate, p = 0.176).

3.4.2 $\delta^{15}N$ signatures of termites and calculation of Δ_{dig} and Δ_{fix}: Unfortunately, the $\delta^{15}N$ signatures of the representative food sources were extremely variable, and all were much higher than is expected for inorganic nitrogen sources (Table 8). Therefore, samples containing the original ammonium nitrate and urea salts were analyzed using IRMS. These analyses yielded values that are more consistent with previously reported $\delta^{15}N$ values: urea = -0.90‰ ± 0.11 (-0.7 ‰; Yun et al., 2006) and ammonium nitrate = -1.5‰ ± 0.04 (-2.3 ‰; Choi et al., 2003). Using these values, Δ_{dig} was calculated to be 1.187, 1.598, and 2.264 for the termites reared on 0% N, 0.1% N urea, and 0.1% N ammonium nitrate, respectively. As Δ_{dig} values for a species fed different diets are likely to be similar (DeNiro and Epstein, 1980), and as the 2.264 value of Δ_{dig} is most consistent with that reported for other termite species (Tayasu, 1998), this value was used in the equation to calculate the $\%N_{dfa}$ within each termite sample.

Additionally, the values for Δ_{fix} were calculated by subtracting the $\delta^{15}N$ signature of non-fixing, antibiotic-treated termites from the $\delta^{15}N$ signatures of the nitrogen-fixing, control termites that were reared on the same N diet (Figure 5). This yielded the anticipated negative values for Δ_{fix} for all feeding treatments except for 1% N urea. In order to eliminate the suppressive effect of dietary N on symbiotic nitrogen fixation, I calculated Δ_{fix} by comparing the $\delta^{15}N$ signatures of antibiotic-treated and control termites that fed on the 0% N diet. This provided a value of Δ_{fix} = -1.245, which is within the range specified for nodulating plants.

3.4.3 $\%N_{dfa}$ versus % dietary nitrogen: By using the calculated values of Δ_{dig} and Δ_{fix}, the $\%N_{dfa}$ within each termite sample was calculated and plotted against the percent nitrogen of the diet on which the termites fed. As Figure 6a illustrates, termites that consumed ammonium

nitrate as their nitrogen source exhibited a strong negative correlation between the amount of nitrogen fixed and the percent of the dietary nitrogen. This negative trend was significant as dietary nitrogen increased from 0% N to 0.21% N ($p = 0.023$), and as dietary nitrogen increased from 0.21% N to 0.94% N ($p < 0.001$).

For termites that fed on urea, the trend was less straightforward. Nitrogen fixation rates were not significantly different between termites exposed to the 0.1% N urea diet and the 0% N diet ($p = 0.927$) (Figure 6b). However, termites reared on 1% N urea demonstrated significantly lower levels of nitrogen fixation than did termites that fed on the 0% N and the 0.1% N urea diets ($p < 0.001$). It is also noteworthy that nitrogen fixation levels were significantly different for termites reared on 0.1% N and 1.0% N urea ($p < 0.001$).

3.5 Discussion

It was surprising that consumption was significantly greater among *R. flavipes* workers that had been treated with antibiotics for three of the five food treatments. As termites were randomly selected from the colony, this result is not attributable to differences in termite size. Perhaps the termites exposed to the antibiotic treatment were experiencing a nutrient deficiency as a result of the loss of their symbionts. However it is not surprising to see that consumption levels were generally higher among termites reared on the low nitrogen food sources relative to those reared on the high nitrogen food sources. As was previously mentioned in Chapter 2, *R. flavipes* does not feed on substrates that are very rich in nitrogen, and therefore it is likely that their microbiota are ill-adapted to metabolize such high nitrogen concentrations. Furthermore, high concentrations of urea and ammonium nitrate may be somewhat toxic to the termites.

Indeed, mortality data also support the conclusion that higher nitrogen substrates could be stressful to termites, as fewer termites survived when fed 1% N urea and 1% N ammonium

nitrate relative to termites reared on the 0% N food sources. Of course, consumption and mortality are related such that replicates with higher levels of mortality would be expected to consume less of the substrate simply because there were fewer termites. In contrast, antibiotic-treated (non-fixing) termites consumed significantly more food than control (nitrogen-fixing) termites, yet both groups experienced similar mortality.

It was fortunate that cannibalism did not differ significantly among the antibiotic and fixing groups of termites for each food substrate, nor did cannibalism differ significantly among termites from the different food treatments ($p = 0.109$). However, the fact that it was present is somewhat problematic as termites themselves represent a much more nitrogen-rich food source than any of the food substrates used in this experiment. Additionally, the observation that consumption levels were generally higher in this experiment than in the previous experiment using natural food sources could be indicative that the cellulose pads were less favorable to termites than the pine wood, pine needles, and aspen leaves that were used in the previous experiment (Chapter 2).

It is problematic that the $\delta^{15}N$ signatures of the food sources were very variable and much higher than those previously reported for inorganic nitrogen sources. To address this difficulty, the sources of urea and ammonium nitrate are currently being reanalyzed with an IRMS, which will likely result in the $\delta^{15}N$ values that are similar to those that were utilized in this study: urea = -0.7‰, and ammonium nitrate = -2.3‰. Although these values are not yet confirmed for the N diets used in this study, using them produced a $\Delta_{dig} = 3.064$, which is consistent with those reported in the literature for other termite species (Taysu, 1998), as well as $\Delta_{fix} = -1.245$, which is within the established range for nodulating plants (Taysu et al., 1994).

Based on the experimentally derived values of Δ_{dig} and Δ_{fix}, a negative correlation between symbiotic nitrogen fixation and dietary nitrogen was apparent. *R. flavipes* workers that consumed 0.1% N or 1% N from ammonium nitrate and 1% N from urea showed a significant reduction in their percentage of nitrogen derived from the atmosphere. In contrast, *R. flavipes* that fed on 0.1% N from urea as the nitrogen source showed a significant increase in symbiotic nitrogen fixation relative to the termites that had consumed 0% N. This is inconsistent with my initial hypothesis, although these results demonstrate that ammonium nitrate is more effective than urea at inhibiting nitrogen fixation in termites. This is likely because of the role that ammonium plays as a negative inhibitor regulating the transcription of the nitrogenase enzyme (Hoover, 2000).

This study again confirms that nitrogen fixation rates within *R. flavipes* are plastic and are responsive to changes in dietary nitrogen. Specifically, these results demonstrate that there is a negative correlation between dietary nitrogen and symbiotic nitrogen fixation. This is contrary to the results obtained by Curtis and Waller (1997) after feeding *Reticulitermes* spp. 1% and 3% solutions of urea (weight to volume). Additionally, these authors failed to find a significant decrease in symbiotic nitrogen fixation in termites that had been fed cellulose pads treated with 1% ammonium nitrate solutions and only reported a significant decrease in nitrogen fixation when termites had been fed on a 5% (weight/weight) treatment. As termites are unlikely to encounter natural substrates that contain this much nitrogen, Curtis and Waller (1997) concluded that it is unlikely that nitrogen concentrations in natural food substrates will ever be high enough to influence symbiotic nitrogen fixation. However, this study demonstrates that even food sources with ~0.1 % N can significantly reduce nitrogen fixation levels, and hence Curtis and Waller's (1997) conclusions may be premature. Indeed, it is likely that the acetylene reduction

assay used in their study was not sensitive enough to determine changes in nitrogen fixation. As the use of stable ^{15}N isotopes is more sensitive to changes in symbiotic nitrogen fixation, this technique may be favored over AR in future studies of nitrogen fixation in termites and other terrestrial arthropods.

However, there are several difficulties associated with stable ^{15}N isotopes, such as challenges obtaining values for Δ_{dig} and Δ_{fix}. While the food sources used in this study need to be reanalyzed to confirm the calculated values of Δ_{dig} and Δ_{fix}, it is impressive that this study identified a way to overcome many of the shortcomings associated with the stable isotope technique. Treating termites with antibiotics yielded reasonable values for both Δ_{dig} and Δ_{fix} in *R. flavipes*. As Δ_{dig} has never been calculated for *R. flavipes*, this represents a significant contribution to what is known about this termite and has great implications for future ecological studies. For example, knowing Δ_{dig} could allow termite researchers to accurately determine food preferences and feeding substrates of *R. flavipes* colonies in the field. Additionally, to the best of my knowledge, Δ_{fix} has never been calculated for any species of termite. This study was able to determine a value for Δ_{fix} that is within the range previously observed for nodulating plants, which will greatly contribute to future studies of nitrogen fixation in termites.

3.6 Conclusion and directions for future study

Future studies should be completed to ensure that the negative relationship between dietary nitrogen and symbiotic nitrogen fixation observed here are conserved, both in termites fed on different nitrogenous substrates and different concentrations, as well as in termites originating from lab and field colonies. Furthermore, field studies should be conducted to identify differences in the nitrogen content and composition of food sources on which *R. flavipes* naturally feeds. The stable isotope technique should be applied to these termites to determine

whether the differences in nitrogen of their natural food substrates are impacting nitrogen fixation rates. Such studies will contribute to our understanding of the amount of atmospheric nitrogen *R. flavipes* contributes to its terrestrial environment, and will allow us to better assess this termite's ecological importance.

Additionally, very little work has been done to determine how symbiotic nitrogen fixation is regulated within termites. For example, do the microbes cease to fix atmospheric nitrogen in the presence of nitrogenous substrates, or do termites control this process by eliminating their N-fixing symbionts or alternatively inhibiting N fixation when they feed on substrates that are high in dietary nitrogen? With the recently developed molecular techniques, such as quantitiative PCR and the quantitative imaging of N fixation developed by Lechene et al. (2007), such studies are possible. Indeed, with so many interesting questions to ask about this ecologically important process, it is not surprising that nitrogen fixation in termites has been an active area of research for decades.

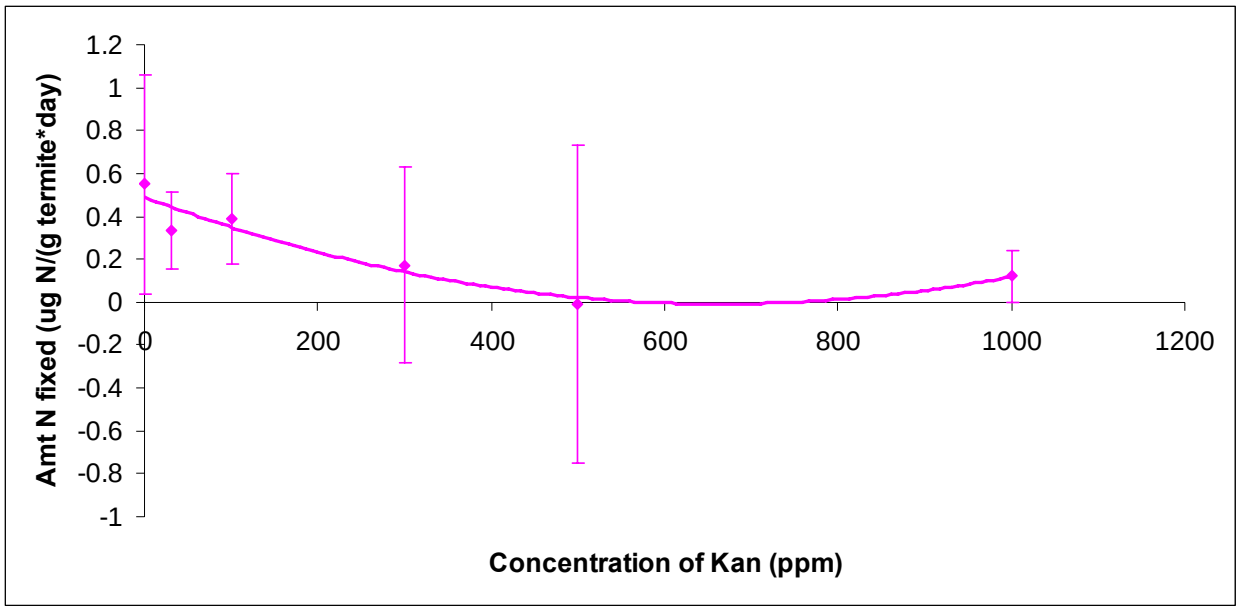

Figure 3: Nitrogen fixation rates in termites (n = 3 reps; 20 termites/rep) exposed *ad lib* to varying concentrations of the kanamicin over a 2-wk feeding period. N fixation was assessed using the acetylene reduction assay. Bars indicate one standard deviation.

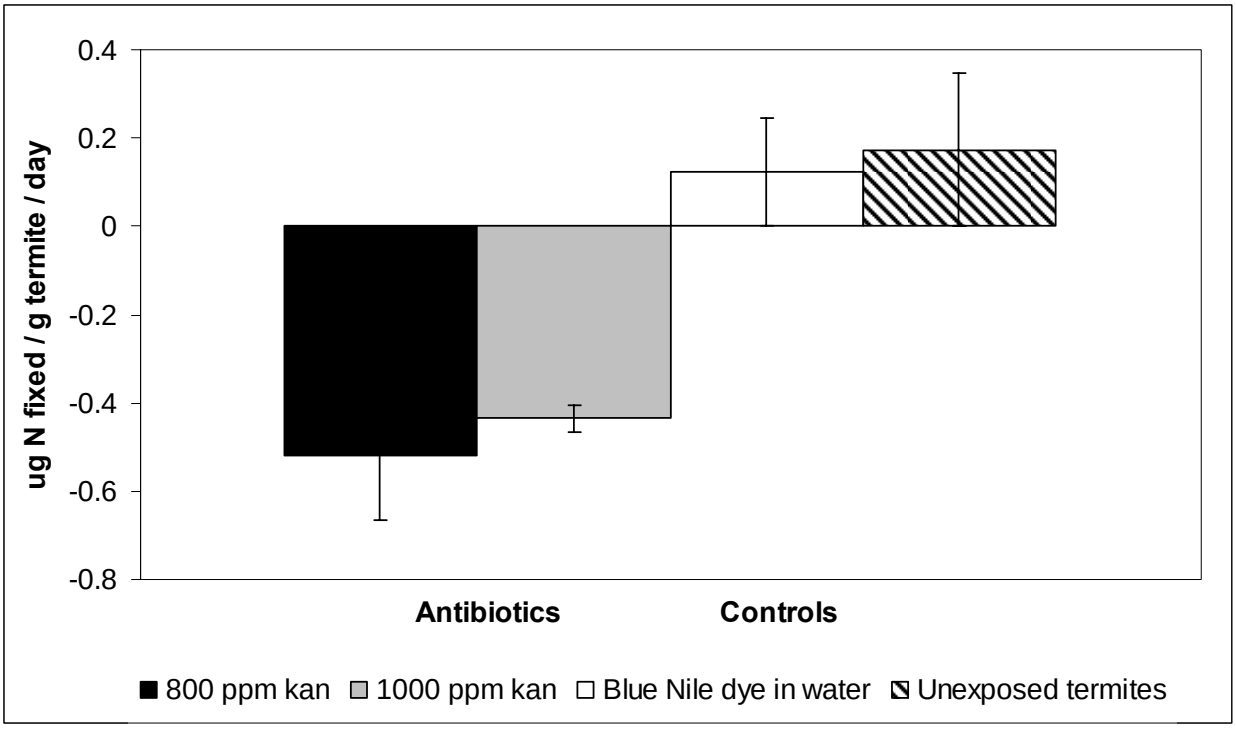

Figure 4: N fixation rates in termites (n = 3 reps; 20 termites/rep) force fed a standardized amount of kanamicin and held for 2 wks. N fixation was assessed using the acetylene reduction assay. Unexposed termites did not receive either an antibiotic or a Nile Blue dye treatment. Bars indicate one standard deviation.

Table 5: Consumption (g) by *R. flavipes* workers reared on each diet (X ± S.E.) for antibiotic-treated and control groups

Treatment	n	Consumption (g) ± S.E.
0% N + antibiotic	4	0.0438 ± 0.001
0% N	5	0.0179 ± 0.002
0.1% N urea + antibiotic	5	0.0353 ± 0.002
0.1% N urea	4	0.0122 ± 0.002
0.1% N ammonium nitrate + antibiotic	4	0.0390 ± 0.065
0.1% N ammonium nitrate	5	0.0201 ± 0.004
1% N urea + antibiotic	4	0.0108 ± 0.002
1% N urea	4	0.0135 ± 0.002
1% N ammonium nitrate + antibiotic	5	0.0184 ± 0.003
1% N ammonium nitrate	3	0.0198 ± 0.003

Table 6: Percent mortality of *R. flavipes* workers (X ± S.E.) reared on each food substrate for antibiotic-treated and nitrogen-fixing groups.

Treatment	n	Average % Mortality ± S.E.
0% N + antibiotic	4	16.07% ± 6.71%
0% N	5	16.43% ± 1.80%
0.1% N urea + antibiotic	5	53.86% ± 11.18%
0.1% N urea	4	64.39% ± 11.05%
0.1% N ammonium nitrate + antibiotic	5	67.86% ± 16.37%
0.1% N ammonium nitrate	5	46.83% ± 10.22%
1% N urea + antibiotic	5	97.36% ± 2.63%
1% N urea	5	80.18% ± 9.56%
1% N ammonium nitrate + antibiotic	5	72.14% ±14.91%
1% N ammonium nitrate	5	46.43% ± 12.22%

Table 7: Percentage of *R. flavipes* workers cannibalized (X ± S.E.) for antibiotic-treated and nitrogen-fixing termite groups reared on each food substrate

Treatment	n	Average % Cannibalized ± S.E.
0% N + antibiotic	4	9.82% ± 2.68%
0% N	5	10.71% ± 1.13%
0.1% N urea + antibiotic	5	32.86% ± 5.22%
0.1% N urea	4	22.66% ± 7.08%
0.1% N ammonium nitrate + antibiotic	5	33.57% ±11.49%
0.1% N ammonium nitrate	5	17.14% ± 2.08%
1% N urea + antibiotic	5	27.32% ± 7.58%
1% N urea	5	10.57% ± 3.71%
1% N ammonium nitrate + antibiotic	5	30.00% ± 10.01%
1% N ammonium nitrate	5	12.86% ± 1.82%

Table 8: $\delta^{15}N$ signatures (X ± S.E.) of each N diet (n = 5)

N Diet	Avg. $\delta^{15}N$ ± S.E.
0% N	0 ± 1.936
0.1% urea	18.15 ± 4.84
0.1% ammonium nitrate	34.01 ± 15.43
1.0% urea	6.62 ± 4.02
1.0% ammonium nitrate	50.86 ± 23.74

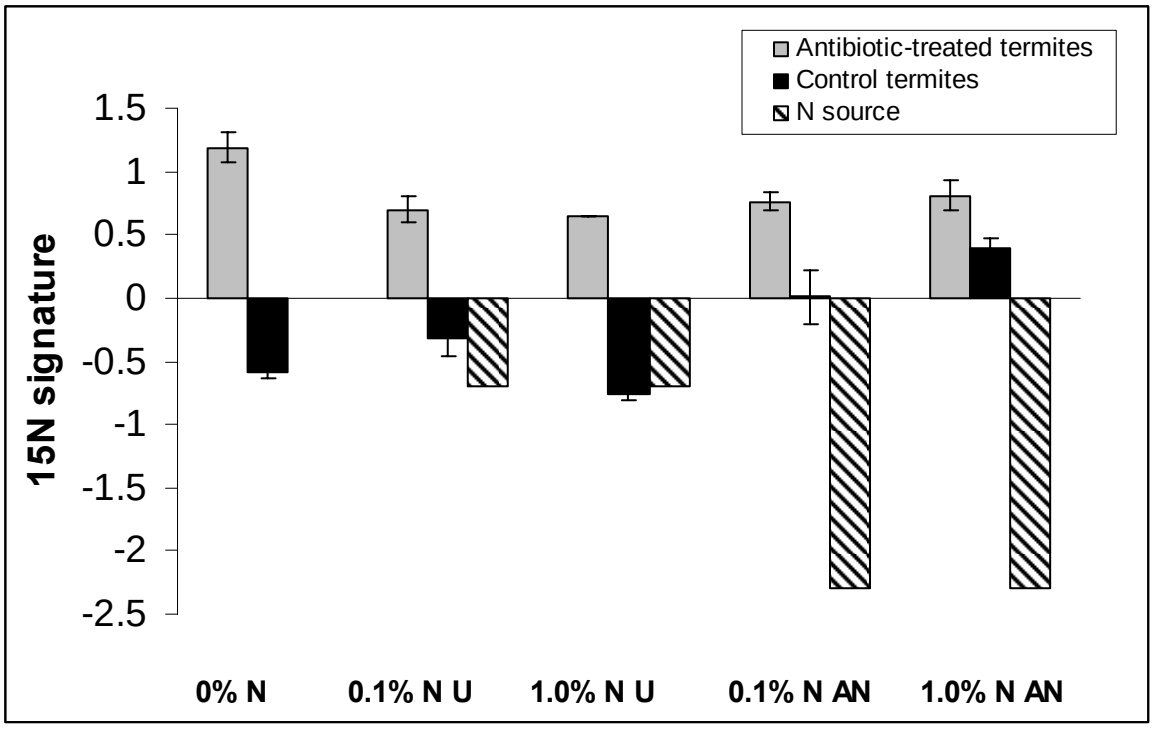

Figure 5: δ^{15}N signature of antibiotic-treated termites, control termites, and their nitrogen source for each nitrogen diet. Here U represents urea and AN represents ammonium nitrate. Bars indicate ± standard error.

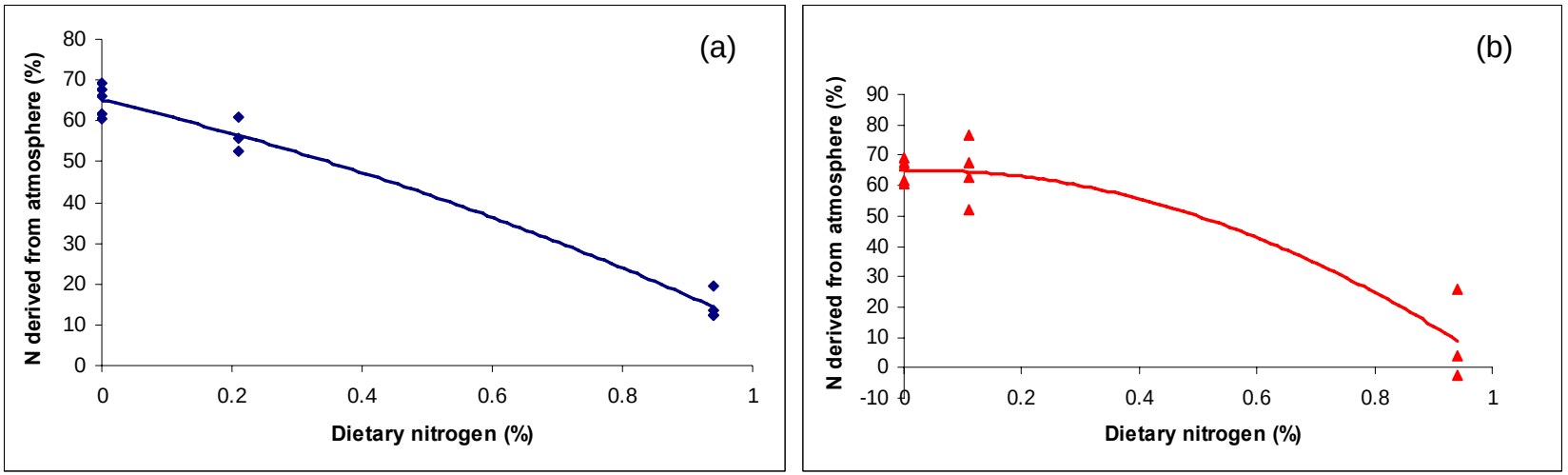

Figure 6: Percent nitrogen derived from the atmosphere (%N_{dfa}) for each termite sample reared on ammonium nitrate as the nitrogen source (a) or urea (b). A.) For termites reared on the ammonium nitrate substrates, the equation that best describes the relationship is $y = -17.569\ x^2 - 37.364\ x + 65.049$ ($R^2 = 0.980$). B.) The exponential equation that best fits this data is $y = -66.789\ x^2 + 3.1175\ x + 65.049$ ($R^2 = 0.896$).

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APPENDIX:

Assessment of Undergraduate Research Experience

I have greatly learned and benefited from my undergraduate research experience in the Department of Entomology. In choosing this individual project I have had the opportunity to explore a topic that greatly interested me. Furthermore, I was fortunate enough to have the assistance of my research advisors, Dr. Susan Jones and Dr. Peter Curtis, in developing clearly defined questions, and experimental designs that were successful in allowing me to make significant discoveries.

However, I also encountered several challenges throughout the course of this project. First among these was the difficulty associated with understanding and correctly applying the equation derived by Tayasu et al. (1994). Initially, I did not understand that the terms Δ_{dig} and Δ_{fix} had not been calculated for termites that have symbiotic N-fixing bacteria, and that these terms would have to be approximated. Fortunately, I was able to estimate these terms in the initial experiment, and then develop a methodology to calculate Δ_{dig} and Δ_{fix} in the second set of experiments.

Another challenge that I encountered was that in successfully inhibiting N fixation in termites. After an extensive literature search, it seemed as if both the antibiotics kanamicin and tetracycline would kill the termite's symbiotic N-fixing bacteria. I therefore fed termites *ad lib* on varying concentrations of each antibiotic, as well as a combination of both kanamicin and tetracycline. My results indicated that only kanamicin was effective, and even then, only at concentrations that were much higher (800-1000 ppm) than those reported (200 ppm) by Matsura et al. (2001) which had successfully eliminated the natural flora of *Reticulitermes speratus*.

Indeed, much of the difficulty that I had in assessing the inhibition of N fixation relates to the challenges that I encountered with the acetylene reduction assay (AR). As AR likely underestimates nitrogen fixation, I realized that there would be difficulties associated with its

use. Therefore I planned only to use AR to confirm the presence or absence of N fixation in termites fed on varying concentrations of antibiotics. However, I discovered that this was not as straightforward as I had hoped. The acetylene I used to conduct these assays was not pure, or lab-grade, and from my analyses, I determined that the gas itself contained significant amounts of ethylene. As AR calculates the amount of N fixation based on the reduction of acetylene to ethylene, this was problematic. To address this difficulty, I determined the amount of background ethylene in samples that contained 1mL of acetylene gas only. I subtracted this background ethylene from the amount of ethylene that was present in samples with termites and the acetylene gas, and I used resulting value to calculate N fixation. In several cases, this gave negative estimates of N fixation, which of course is impossible. This problem easily could have been avoided if I had been able to obtain lab-grade acetylene, but unfortunately I was unsuccessful, and some of my results suffered.

Yet these challenges were not entirely negative, as they allowed me to grow as a more independent and autonomous researcher. I learned how to identify the causes of the problems I encountered, and I was often able to discover ways to overcome them. As any good researcher needs to have the tenacity to prevail in spite of difficulties, as well as the ability to design troubleshooting techniques, I feel that these experiences have been invaluable to my development as a scientific researcher.

Furthermore, no discussion of the personal growth my undergraduate research has afforded me would be complete without addressing how I have benefited from scientific collaboration. This project has given me the opportunity to work with scientists from different backgrounds, such as entomology, ecology, and chemistry. Through working with researchers with such diverse areas of expertise, my knowledge of termite biology, and stable isotopes has

been greatly enhanced. Additionally, I have been fortunate enough to have the assistance of faculty and staff from Ohio State's Microbiology, and Horticulture and Crop Science Departments, as well as other members of the Entomology and EEOB Departments who were not associated with either Dr. Susan Jones or Dr. Peter Curtis' labs. Through working with these researchers I have developed a variety of techniques such as antibiotic preparation and application, AR, using and calibrating a microbalance, and preparing samples with a Wiley Mill. This project has taught me firsthand that science is truly a collaborative effort that profits through working with people from different disciplines and backgrounds. I greatly appreciate all those who helped to make this research project possible, and I look forward to engaging in collaborative research in the future.